The protein tyrosine phosphatase SHP-1 regulates integrin-mediated adhesion of macrophages

Tamara I.A. Roach*, Suzanne E. Slater*, Lynn S. White†, Xiaoling Zhang‡, Philip W. Majerus‡, Eric J. Brown* and Matthew L. Thomas†

The Src homology 2 domain phosphatase-1 (SHP-1) is a tyrosine phosphatase containing two amino-terminal SH2 domains and is expressed primarily by hematopoietic-derived cells [1]. The *viable motheaten* (*Hcph*$^{me\text{-}v}$) mutant mice (*me*^{v}) suffer from progressive inflammation due to a deficiency of SHP-1 enzyme activity [2,3] and die at 3–4 months of age from macrophage and neutrophil accumulation in the lung [4]. The mechanism by which SHP-1 deficiency leads to inflammation is unknown. We found that macrophages from *me*^{v} mice adhered and spread to a greater extent than normal macrophages through αmβ2 integrin-mediated contacts. Whereas macrophages deficient in the transmembrane tyrosine phosphatase CD45 (*CD45*$^{-/-}$) spontaneously detached from αmβ2 integrin contacts [5], cells deficient in both CD45 and SHP-1 did not. In SHP-1-deficient macrophages there was a 10–15-fold increase in D-3 phospholipid products of phosphatidylinositol (PI) 3-kinase. Concomitantly, there was a 2–5-fold increase in membrane-associated PI 3-kinase activity in *me*^{v} macrophages relative to normal macrophages. Treatment of macrophages with the PI 3-kinase inhibitors wortmannin or LY294002 resulted in a dramatic detachment of cells, indicating that PI 3-kinase activity is required for adhesion. These data demonstrate that SHP-1 is necessary for detachment from αmβ2 integrin-mediated contacts in primary macrophages and suggest that a defect in this pathway may contribute to inflammatory disease.

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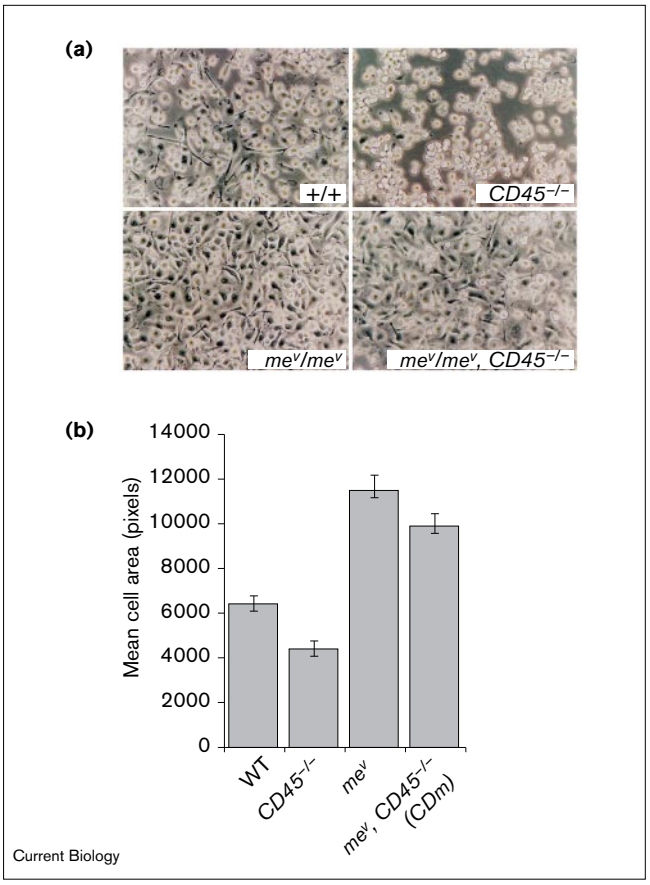
Results and discussion

To investigate whether SHP-1 can regulate αmβ2 integrin-mediated adhesion, bone marrow derived macrophages from normal, *CD45*$^{-/-}$, *me*^{v} mice or mice deficient in both CD45 and SHP-1 (*CDm*) were replated and allowed to re-adhere, an event dependent upon αmβ2 integrin adhesion [5,6]. After 24 hours, the majority of mature macrophages from normal mice were adherent; however, a substantial fraction were refractile with minimal spreading (Figure 1a). As we reported previously [5], CD45-deficient macrophages were almost entirely detached due to accelerated de-adhesion (Figure 1a). In contrast, macrophages from both *me*^{v} and *CDm* mice were adherent and markedly spread (Figure 1a). Quantitation of adherent cell areas of single cells plated at low density revealed that CD45-deficient macrophages were significantly less spread than normal macrophages (Figure 1b). However, both *me*^{v} and *CDm* cells showed significantly increased adhesion and spreading when compared with normal macrophages (Figure 1b). This demonstrates that catalytically active SHP-1 is required for the previously reported spontaneous de-adhesion observed in CD45-deficient macrophages [5]. Compared with normal macrophages, it is likely that the increased spreading in *me*^{v} and *CDm* macrophages reflects perturbation of a dynamic balance between adhesion and de-adhesion which requires SHP-1. Furthermore, the data suggest that SHP-1 phosphatase activity is necessary for and downstream of CD45's regulation of adhesion.

The p85/p110 PI 3-kinase can be activated by integrin adhesion [7–9] and can regulate cytoskeletal rearrangement controlling cell spreading and adhesion [10,11]. The PI D-3 lipids, PI 3,4-bisphosphate, (PI(3,4)P_2) and PI 3,4,5-trisphosphate (PI(3,4,5)P_3) are the main products of PI 3-kinase activation *in vivo* [12]. Hence, to determine whether PI 3-kinase activity was deregulated in the absence of catalytically active SHP-1, we examined levels of PI lipids produced by adherent normal, CD45-deficient, *me*^{v} and *CDm* macrophages following metabolic labeling of cells with $^{32}[P_i]$. When normalized to total phospholipid content, *me*^{v} and *CDm* macrophages exhibited more than 10–15-fold higher levels of D-3 phospholipids (Figure 2a), indicating a higher level of PI 3-kinase activity at the membrane. This was not due to different expression patterns of the p110α, β or γ subunits in the macrophages of different genotypes (data not shown). In contrast, levels of PI and PI(4,5)P_2 were similar to normal in *me*^{v} and *CDm* macrophages (Figure 2).

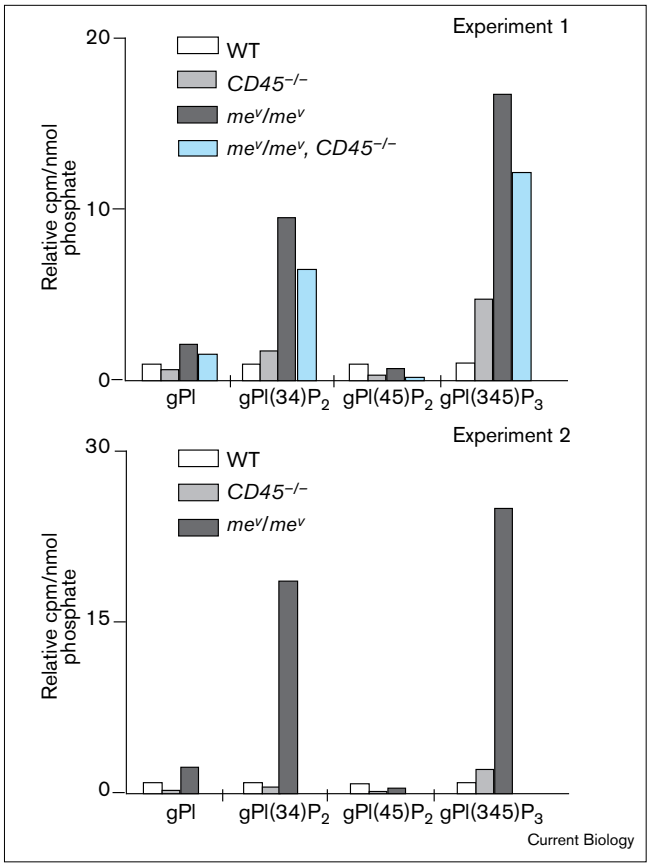
To determine whether the increased adhesion and spreading observed in *me*^{v} or *CDm* macrophages was dependent upon PI 3-kinase activity, macrophages were treated with wortmannin, an inhibitor of PI 3-kinase [13] (Figure 3a). Low concentrations of wortmannin (20 nM) led to de-adhesion of

Figure 1



Adhesion of bone marrow derived macrophages from normal (+/+, WT), CD45-deficient (*CD45*$^{-/-}$), *me*^{v}, or *CD45*$^{-/-}$ *me*^{v} (*CDm*) mice to αmβ2 ligand. **(a)** Mature bone marrow derived macrophage cultures were harvested (PBS–10 mM EDTA) and allowed to re-adhere in medium on non-tissue culture plastic, an αmβ2 ligand [5,6], for 24 h. Photographs were taken at magnification 320 ×, scanned into Adobe photoshop 3.0 (Adobe Systems) at 300 dots per inch, and cell area quantitated using Image Pro Plus (Media Cybernetics). **(b)** Quantitation of macrophage adhesion per area of cell spread. Macrophages were harvested from adherent cultures using PBS–10 mM EDTA and allowed to re-adhere at low density in medium for 24 h. Columns in (b) show means ± SEM; numbers of cells measured were 149, 46, 98 and 93 for WT, *CD45*$^{-/-}$, *me*^{v} and *CDm,* respectively. *CD45*$^{-/-}$, *me*^{v} and *CDm* cell areas were all significantly different from WT (Student's *t* test, $p < 0.05$).

Figure 2



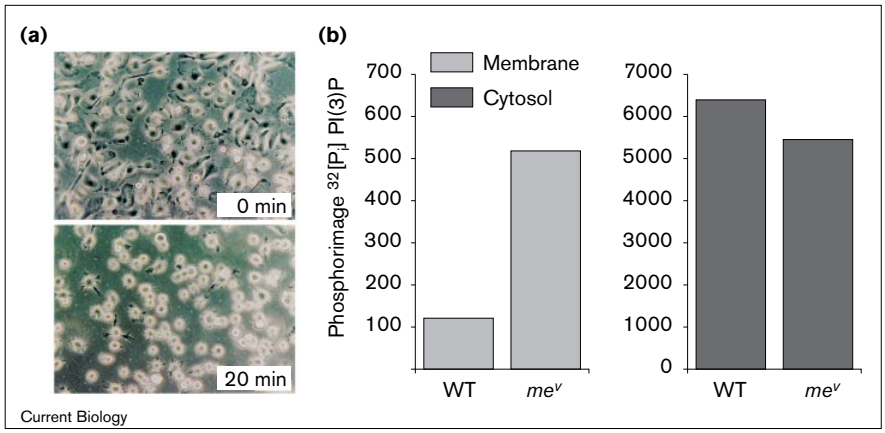
Increase in PI 3-kinase activity in *me*^{v} macrophages *in vivo* correlates with increased adhesion. PI D-3 lipids are elevated in bone marrow derived macrophages from either *me*^{v}, or *CD45*$^{-/-}$ *me*^{v} (*CDm*) mice. Bone marrow derived macrophages from either normal (WT), *CD45*$^{-/-}$, *me*^{v} or *CDm* were metabolically labeled with $^{32}[P_i]$ for 4 h, lysed in acid and the lipids extracted. Lipids were separated by column chromatography and normalized to total organic phosphate. The results of two experiments are shown. Abbreviations: gPI, glycerophosphoinositol monophosphate; gPI(34)P_2, glycerophosphoinositol 3,4-bisphosphate; gPI(45)P_2, glycerophosphoinositol 4,5-bisphosphate; gPI(343)P_3, glycerophosphoinositol 3,4,5-trisphosphate.

macrophages of all genotypes within 1 hour (Figure 3a and data not shown). LY294002, a PI 3-kinase inhibitor with a mode of action distinct from that of wortmannin [14], also induced de-adhesion at 20 μM (data not shown). These data show that maintenance of PI 3-kinase activity is required for continued macrophage adhesion and support the conclusion that SHP-1 regulation of PI 3-kinase is important for its modulation of macrophage adhesion and spreading.

Membrane localization of PI 3-kinase is required for enzyme activity because translocation of PI 3-kinase allows access to phospholipid substrate, and interactions of the p85 regulatory subunit with other proteins may regulate a conformational activation of the p110 catalytic subunit [12,15]. PI 3-kinase immunoprecipitated (by anti-p85) from normal and *me*^{v} whole-cell lysates showed similar activities in *in vitro* assays (data not shown). However, fractionation of the cells into membrane and cytosol and immunoprecipitation with anti-p85 revealed that *me*^{v} macrophages had a 2–5-fold increase in the proportion of PI 3-kinase activity associated with membranes (Figure 3b and data not shown).

The *me*^{v} disease is characterized by excessive idiopathic inflammation as well as increased production of myeloid

Figure 3



PI 3-kinase activity is required for integrin-mediated adhesion in macrophages and is increased in the membrane fraction of cells. **(a)** *CDm* bone marrow derived macrophages before (top) and after (bottom) treatment with wortmannin. Adherent macrophages were treated for 20 min with 20 nM wortmannin at 37°C. Magnification is 320×. Similar results were obtained for macrophages from the other genotypes. The PI 3-kinase inhibitor LY294002 (20 μM) caused a similar rounding and detachment of cells. **(b)** PI 3-kinase was immunoprecipitated from membrane and cytosol fractions of wild-type (WT) and me^v macrophages (1×10^7 cells) with anti-p85 antisera and its activity assayed *in vitro*. $^{32}[P_i]$ activity incorporated into the PI substrate by PI 3-kinase was quantitated by phosphorimage analysis after thin-layer chromatography separation of lipids. PI 3-kinase activity in the membrane fraction relative to total cell activity for WT and me^v macrophages was 1.83% and 9.84%, respectively.

cells, including extramedullary hematopoiesis. The unregulated inflammation leads to skin disease, arthritis and pneumonitis, eventually ending in death of the animal. Inflammation can be inhibited by blocking αmβ2, the predominant myeloid integrin, suggesting that regulation of adhesion has a prominent role in me^v disease [16]. We found that deficiency of SHP-1 induces enhanced αmβ2-mediated adhesion, regardless of whether or not CD45 is expressed by the macrophages. The spontaneous detachment from αmβ2-mediated contacts that occurs to some extent in normal macrophages, and is dramatically increased in the absence of CD45, is abrogated by a deficiency of SHP-1. As me^v mice express a catalytically impaired SHP-1 protein, this finding demonstrates that SHP-1 phosphatase activity is required for normal regulation of macrophage de-adhesion.

The mechanism by which SHP-1 activates de-adhesion probably requires that it regulate PI 3-kinase. The me^v and *CDm* macrophages have markedly increased D-3 phospholipid levels, indicating enhanced PI 3-kinase activity *in vivo*, and PI 3-kinase inhibitors cause detachment of normal and SHP-1-deficient macrophages from αmβ2-integrin-mediated contacts. Because total cellular PI 3-kinase activity is not affected by SHP-1 deficiency, but membrane-associated PI 3-kinase is markedly increased in its absence, it is specifically the membrane-associated pool of PI 3-kinase which is regulated by SHP-1. Although this is a minority of whole-cell PI 3-kinase protein, it is probably the physiologically important pool of enzyme as it is associated with substrate.

Mice deficient in the SH2-containing inositol phosphatase SHIP have a remarkably similar phenotype to me^v mice, with an accumulation of macrophages and neutrophils in the lung and a shortened life span [17]. It has been shown in cell lines that inhibitory signaling following specific receptor recruitment of SHIP or SHP-1 can function via independent receptor pathways [18]. Our data suggest that SHP-1 regulates the level of PI D-3 lipids through the control of PI-3 kinase activation. Thus, pathways inhibited by SHP-1 and SHIP may converge at the level of regulation of phosphorylated phosphatidylinositol lipids. The similar phenotypes of me^v mice and SHIP-deficient mice suggest that it is deregulation of phosphatidylinositol phosphorylation that contributes significantly to the idiopathic inflammation in both mouse strains.

CD45 and SHP-1 have been proposed to have opposing effects on lymphocyte activation [19]. If the same were true for myeloid activation, or if the inflammatory disease of me^v mice was due to a primary abnormality in lymphocyte signaling, CD45 deficiency should ameliorate the disease of me^v mice. However, *CDm* mice present a disease pathology indistinguishable from that of me^v mice, with involvement of skin, thymus, spleen, kidney and lungs (unpublished observations). Together with data demonstrating that a blockade of αmβ2 interaction slows the me^v disease in a bone marrow chimeric model [16], these data strongly suggest that the failure to regulate macrophage de-adhesion normally may be critical for the spontaneous inflammation that characterizes the me^v disease. We propose that the pathologic accumulation of leukocytes in lung and other tissues of me^v and *CDm* mice results in part from the failure to properly regulate leukocyte de-adhesion. Normal migration of phagocytes into tissues is accompanied by integrin activation and enhanced adhesion, a reversible process [20–22]. After migration of macrophages out of the vasculature and into tissue at sites of inflammation, the regulation of macrophage adhesion is likely to be key to the control of macrophage survival and activation at these sites. The failure of me^v and *CDm* macrophages to

regulate adhesion and spreading appropriately, as shown in our *in vitro* studies, and the hyperactivation of PI 3-kinase, could be central to the *in vivo* accumulation of macrophages in tissue in SHP-1-deficient mice [23]. In conclusion, these studies have elucidated a novel function for SHP-1 — the regulation of integrin-mediated adhesion through regulation of the function of PI 3-kinase. Pathologic deregulation of this interaction leads to inflammation and is a likely biochemical target for intervention to control the idiopathic and excessive inflammation that characterizes human autoimmune diseases.

Supplementary material

Additional methodological details are published with this paper on the internet.

Acknowledgements

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Supplementary material

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Materials and methods

Mice

All mice were maintained according to NIH guidelines in a specific pathogen-free barrier facility. Breeding pairs of *me^v* mice were purchased from Jackson Laboratory and maintained in our facility for greater than 3 years by crossing heterozygous pairs. The presence of the *me^v* allele was determined by PCR as previously described [S1]. The derivation of the CD45-deficient animals has been described previously [S2]. CD45-deficient mice were backcrossed to B6 mice for at least nine generations and maintained as homozygous mice. To generate mice deficient in both SHP-1 and CD45, *me^v* mice were crossed to CD45-deficient mice to generate mice heterozygous for each allele. Doubly heterozygous mice were crossed to *CD45$^{-/-}$* mice to generate mice that were heterozygous for the *me^v* allele and homozygous for the CD45-gene targeted allele. *CDm* mice were generated by crossing heterozygous *me^v*, CD45$^{-/-}$ mice. Mice were from 4 to 12 weeks of age, and were sex- and age-matched (within 2 weeks of age) within experiments.

Adhesion

Macrophages were differentiated from bone marrow precursors and maintained in culture as previously described [5]. Briefly, bone marrow derived macrophages were differentiated from promonocytes plated at 5×10^8 to 1×10^7 cells per dish on non-tissue culture treated plastic (Valmark 'ultradish', Midwest Scientific) in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL), with 10% fetal bovine serum (FBS; HyClone Labs), 20 mM HEPES, and 15% L929 cell-conditioned supernatant as a source of M-CSF. Mature bone marrow macrophages were harvested from day 6 to day 15 in PBS containing 10 mM EDTA. Wortmannin and LY294002 were dissolved in DMSO and used at final concentrations of 1–20 nM or 5–20 μM, respectively.

Immunoprecipitations and kinase reactions

Macrophages, $3–8 \times 10^6$ cells per point, were lysed in ice cold Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl pH 7.5) containing 1% Triton X-100 (Pierce), 1% deoxycholate, 1 mM EDTA (lysis buffer), with 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM NaF, 200 μM pervanadate and 2 mM diisopropyl-fluorophosphate (all chemicals from Sigma). Nuclei and debris were removed by centrifugation and supernatants collected. Lysates were precleared with protein A (or G)–Sepharose coupled to normal rabbit IgG or whole serum. Immunoprecipitations were performed using protein A/G coupled to rabbit IgG against SHP-1 or whole rabbit serum anti-p85 (#06-195, UBI) or SHP-1 [S3]. Immunoprecipitates collected by centrifugation were washed three times in lysis buffer with inhibitors and resolved by SDS–PAGE.

Kinase activity of immunoprecipitated p85 was determined by an *in vitro* kinase assay using phosphotidylinositol as substrate (Sigma or Avanti Polar Lipids). After washing (twice in 10 mM Tris pH 7.5/150 mM NaCl/1% Triton X-100, twice in 10 mM Tris pH 7.5/0.5 M LiCl, twice in PI 3-kinase reaction buffer; see below), p85 was resuspended in PI 3-kinase reaction buffer (20 mM HEPES pH 7.4, 0.4 mM EGTA, 10 mM $MgCl_2$, 100 μM pervanadate) with PI substrate. To initiate the kinase reaction 10 μCi [γ-^{32}P]ATP (4500 Ci/mM, ICN Biomedicals) and unlabeled ATP (20 μM final concentration) were added. Reactions were stopped by adding 80 μl 1N HCl and 160 μl chloroform:methanol (1:1). Lipids were extracted twice, then resolved on thin-layer chromatography plates (Whatman) coated with potassium oxalate.

Analysis of D-3 phosphate-containing inositol phospholipids

Differentiated macrophages were labeled with 1 mCi/ml $^{32}[P_i]$ (400–800 μCi/ml, HCl free (ICN Biomedicals) in phosphate-free DMEM, 5% fetal calf serum for 4 h at 37°C. D-3 phosphate-containing inositol phospholipids were analyzed as previously described [S4]. Briefly, monolayers were lysed in 1 M perchloric acid and lipids extracted with $CHCl_3$: methanol (1:2, v/v) and deacylated with methylamine. Glycerophospholipids were separated by HPLC using a Parsil 10 Sax column (Whatman) [S5]. [^{3}H]inositol 1-monophosphate, [^{3}H]inositol 1,4-bisphosphate, and [^{3}H]inositol 1,3,4-trisphosphate were used as internal standards in HPLC. [^{32}P]3-phosphate-containing glycerophosphoinositols, [^{32}P]gPI 3-P, [^{32}P]gPI 3,4-P_2, [^{32}P]gPI 3,4,5-P_3 were prepared as described [S6], chromatographed separately and used to identify specific glycerophosphoinositols.

Membrane/cytosol fractionation

Macrophages were washed and resuspended in ice cold sonication buffer (20 mM HEPES pH 7.4, 0.5 mM pervanadate, 1 mM DFP, 0.5 mM NaF, 10 μg/ml leupeptin/aprotinin, 1 mM pepstatin A). Cells were sonicated on ice using a sonication probe (Vibra cell, Sonics and Materials). Nuclei and coarse debris were removed by centrifugation (650 *g*, 15 min). Supernatants were collected and centrifuged at 100,000 *g* (1 h) to separate membrane/cytoskeleton from cytosol. Membrane fractions were resolubilized in 1% Triton X-100/1% deoxycholate plus inhibitors in lysis buffer (see above) and sonicated, insoluble matter was removed by centrifugation (15,000 *g*, 10 min). Detergents and NaCl were added to cytosol preparations at equivalent concentrations for immunoprecipitations.

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